J. Membrane Biol. 102, 97–104 (1988)

The Journal of
Membrane Biology

Cadmium Inhibits Plasma Membrane Calcium Transport

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Summary. The interaction of Cd^{2+} with the plasma membrane Ca^{2+}-transporting ATPase of fish gills was studied. ATP-driven Ca^{2+}-transport in basolateral membrane (BLM) vesicles was inhibited by Cd^{2+} with an I_{50} value of 3.0 nM at 0.25 μM free Ca^{2+}, using EGTA, HEEDTA and NTA to buffer Ca^{2+} and Cd^{2+} concentrations. The inhibition was competitive in nature since the $K_{0.5}$ value for Ca^{2+} increased linearly with increasing Cd^{2+} concentrations while the V_{max} remained unchanged. The Ca^{2+} pump appeared to be calmodulin dependent, but we conclude that the inhibition by Cd^{2+} occurs directly on the Ca^{2+} binding site of the Ca^{2+}-transporting ATPase and not via the Ca^{2+}-binding sites of calmodulin. It is suggested that Cd^{2+}-induced inhibition of Ca^{2+}-transporting enzymes is the primary effect in the Cd^{2+} toxicity towards cells followed by several secondary effects due to a disturbed cellular Ca^{2+} metabolism. Our data illustrate that apparent stimulatory effects of low concentrations of Cd^{2+} on Ca^{2+}-dependent enzymes may derive from increased free-Ca^{2+} levels when Cd^{2+} supersedes Ca^{2+} on the ligands.

Key Words BLM vesicles · Ca^{2+} transport · Cd^{2+} inhibition · calmodulin · trout gills

Introduction

Exposure of fish to cadmium (Cd) in the water is known to cause a wide spectrum of toxic effects (Christensen, 1975; Benoit et al., 1976; McCarty & Houston, 1976; Webb, 1979; Bishop & McIntosh, 1981; Majewski & Giles, 1981). Fish exposed to this heavy metal become hypocalcemic (Roch & Maly, 1979; Giles, 1984). Disturbed ionoregulation, as a result of the reduced plasma Ca^{2+} level, has been implied as the fundamental mechanism of Cd^{2+} toxicity (Roch & Maly, 1979; Giles, 1984).

It is well established that for Ca^{2+} homeostasis freshwater fish depend on their gills as the primary site for Ca^{2+} uptake from the water (Berg, 1970; Payan & Matty, 1975; Milet, Peignoux-Deville & Martelly, 1979; Ichii & Mugiya, 1983; Flik, Van Rijs & Wendelaar Bonga, 1985*a*). The uptake of Ca^{2+} from the water is considered the resultant of an energy-dependent transcellular Ca^{2+} inflow and a passive paracellular Ca^{2+} efflow over the branchial epithelium (Flik, Wendelaar Bonga & Fenwick, 1984; Flik et al., 1985*a*; Verbost et al., 1987*a*). It was shown before that transepithelial Ca^{2+} inflow in fish gills depends on a high-affinity Ca^{2+}, Mg^{2+}-ATPase activity (Flik et al., 1985*a*). It seems reasonable to postulate that Cd^{2+}-induced hypocalcemia is a direct consequence of an impaired branchial exchange. This postulate is supported by our recent observations (Verbost et al., 1987*a*) that Cd in the water (5×10^{-8} to 1×10^{-7} M) inhibits specifically Ca^{2+} inflow in trout gills. The same concentrations of waterborne Cd did not affect Ca^{2+} efflow. From these experiments, and others in which we evaluated entry of Ca^{2+} into the epithelium, we tentatively concluded that ATP-driven Ca^{2+} transport over the basolateral membrane becomes inhibited when fish are exposed to Cd. Assuming that the trout branchial Ca^{2+} pump is calmodulin dependent as reported for Ca^{2+}-ATPase in other membrane systems (Larsen & Vincenzi, 1979; Lynch & Cheung, 1979), one may predict at least two possible modes of interaction between Cd^{2+} and the Ca^{2+}-ATPase, viz. indirectly via calmodulin and directly via the Ca^{2+} site of the enzyme.

For a variety of other calmodulin-stimulated enzymes it has been shown that Cd exerts a biphasic effect, i.e. stimulation at low concentrations and inhibition at high concentrations (Mazzei, Girard & Kuo, 1984; Suzuki et al., 1985) and these effects were suggested to be mediated through its binding to calmodulin. Flik et al. (1987) concluded that Cd^{2+} inhibits phosphodiesterase activity in two ways, viz. directly via the enzyme and indirectly via interaction with its activator calmodulin. Akerman et al. (1985) reported on the influence of Cd on Ca^{2+}, Mg^{2+}-ATPase in erythrocyte ghosts and concluded that Cd^{2+} may exert an inhibitory effect on the Ca^{2+}-ATPase by the interaction with the regulatory calmodulin. From a recent study by our group (Verbost, Senden & Van Os, 1987*b*) we concluded that

Table 1. Relative recoveries and purification of marker enzymes in trout gill plasma membranes[a]

	H_0V_{spec}[b]	P_3V_{spec}[c]	% recovery	Enrichment[d]
Protein	—	—	1.5 ± 0.3	—
Na^+,K^+-ATPase	3.2 ± 1.6	33.7 ± 3.5	13.0 ± 2.8	10.5
SDH	39.4 ± 7.3	17.1 ± 10.1	0.7 ± 0.4	0.4

[a] Mean values ± SE of six to nine different experiments are given.
[b] V_{spec} = μmol P_i/hr · mg protein, at 37°C.
[c] $V_{spec} = A_{490}$/min · mg protein, at 25°C.
[d] Enrichment = $V_{spec}P_3/V_{spec}H_0$.

Cd^{2+} inhibits Ca^{2+} transport in rat intestine basolateral membrane vesicles in a competitive way, directly on the Ca^{2+} binding site of the Ca^{2+}-ATPase. In an attempt to get these different results on one line we started this study.

We report on the effects of Cd^{2+} on the kinetics of Ca^{2+} transport in resealed vesicles of isolated basolateral membranes (BLM) from gill epithelium. We postulate that inhibition of the gill Ca^{2+}-ATPase by Cd^{2+} occurs directly on the Ca^{2+} site of the enzyme and not via interaction of Cd^{2+} with calmodulin. Furthermore, it will be shown that in media buffered for Ca^{2+} (and Cd^{2+}) it is actually the rise in free Ca^{2+} upon addition of low concentrations of Cd^{2+} that underlies the apparent stimulatory effects of Cd^{2+} both in our assay system and in data from the literature that were reanalyzed according to this notion.

Materials and Methods

Fish

Male and Female rainbow trout *Salmo gairdneri* with an average body weight of 165 g were obtained from a commercial dealer in Beek, The Netherlands. In the laboratory trout were kept in running Nijmegen tapwater (0.8 mM Ca^{2+}, 10°C) under a photoperiod of 12-hr light per day. The animals were fed trout pellets (Trouvit).

Isolation of Plasma Membranes

Plasma membranes of branchial epithelium were isolated as described in detail by Flik, Wendelaar Bonga and Fenwick (1985*b*). In short, after homogenization (2-min ultraturrax) of gill epithelial scrapings, nuclei and cellular debris (pellet, P_0) were separated from membrane fractions (supernatant, depicted as H_0) by centrifugation at 550 × *g* for 10 min. Next membranes were collected by ultracentrifugation of H_0 (50 Krpm, 30 min, Beckman Ti 70 rotor) and a pellet (P_1) consisting of a firm brownish part with a fluffy layer on top was obtained. This fluffy layer was resuspended in isotonic sucrose-buffer with a Dounce homogenizer (100 strokes). This membrane suspension was centrifuged differentially: 1000 × *g* for 10 min, 10,000 × *g* for 10 min (yielding P_2) and 50,000 × *g* for 30 min (Sorval RC-5B) yielding the final pellet P_3.

Plasma membrane vesicles obtained were resuspended by 10 passages through a 23-G needle in a buffer containing 20 mM HEPES/Tris (pH 7.4), 1.5 mM $MgCl_2$ and 150 mM KCl (Ca^{2+}-transport studies) or 150 mM NaCl (enzyme studies). From the branchial apparatus of a 165 g trout a crude homogenate (H_0) containing 89.6 ± 24.2 mg protein (n = 17) was obtained: 1.35 ± 0.46 mg protein (n = 17) was recovered in the BLM vesicle fraction. Membrane preparations were used on the day of isolation without being frozen.

Calmodulin Depletion Studies

Referring to the steps of membrane isolation, for calmodulin depletion the fluffy layer of P_1 was resuspended in 5 mM EGTA containing isotonic sucrose buffer (in controls isotonic sucrose buffer without EGTA was used). The very low Ca^{2+} concentration thus obtained decreases the affinity of calmodulin for the Ca^{2+}-ATPase (Foder & Scharff, 1981) and enables separation by centrifugation of the calmodulin dissociated from the membranes. The degree of calmodulin depletion was determined with a calmodulin RIA (Amersham, code IM.150).

Enzyme Assays

Routinely, two marker enzymes were used to characterize the membrane preparations, *viz.* Na^+,K^+-ATPase for basolateral plasma membranes and succinic acid dehydrogenase (SDH) for mitochondrial fragments (for assay conditions *see* Flik, Wendelaar Bonga & Fenwick, 1983). Maximum enzyme activities were obtained after preincubation (10 min at 37°C) with detergent at optimal concentration: 0.20 mg · ml^{-1} saponin was used at a protein concentration of about 0.50 mg · ml^{-1}.

Protein was determined with a commercial reagent kit (Biorad), with bovine serum albumin (BSA) as reference. Data on recovery and purification of Na^+,K^+-ATPase and SDH activities in trout branchial epithelium membrane fractions in our isolation procedure are given in Table 1. The final membrane fraction used was enriched 10.5 times in the BLM marker Na^+,K^+-ATPase, as compared to the initial tissue homogenate (H_0). Only a small contamination with mitochondrial membrane fractions was present in this preparation as indicated by a purification factor of 0.4 for succinic acid dehydrogenase (SDH) activity. To exclude contributions of mitochondrial membranes 5 μg · ml^{-1} oligomycin B was routinely included in the Ca^{2+}-transport assay media. The aforementioned factors for purification

and recovery are in line with previously published data on eel (Flik et al., 1985*b*) and with a recent study on trout (Perry & Flik, 1987). It has been shown for identical membrane preparations that this isolation procedure results in low purification factors and recoveries for thiamine pyrophosphatase, NADH- and NADPH-dependent cytochrome *c* reductase (Perry & Flik, 1987).

Vesicular Space

Uptake of D-(^{14}C)-mannitol (Amersham International) in membrane vesicles was measured in the Ca^{2+}-transport medium (10^{-6} M Ca^{2+}, without ^{45}Ca) to which 100 μM mannitol plus 7.6×10^5 Bq · ml^{-1} ^{14}C-mannitol had been added.

The vesicular space for trout gill plasma membranes calculated on the basis of vesicle mannitol content at equilibrium at 1 hr was 6.70 ± 2.32 μl · mg^{-1} protein ($n = 8$), a value comparable to the one reported for vesicles obtained from eel gill plasma membranes [2.21 μl · mg^{-1} protein (Flik et al., 1985*b*)]. Calmodulin depletion by EGTA-treatment did not affect the vesicular space (*results not shown*).

Ca^{2+}-Transport Studies

ATP-dependent Ca^{2+} transport was determined by means of a rapid filtration technique as described by Van Heeswijk, Geertsen and Van Os (1984). The composition of the assay medium (final concentrations in mM) is: HEPES/Tris (20, pH 7.4), Tris-ATP (3), KCl (150), free Mg^{2+}(1.5), EGTA (0.5), HEEDTA (0.5), NTA (0.5). Oligomycin B was added in a concentration of 5 μg · ml^{-1}. All incubations were carried out at 37°C. The free Ca^{2+}- and Cd^{2+}-concentrations (mentioned in the Tables and Figures) at the various conditions were calculated according to van Heeswijk et al. (1984). Stability constants of the ligands (EGTA, HEEDTA, NTA and ATP) were obtained from Sillen and Martel (1964). We determined the association constant K for Cd-ATP in a Cd^{2+}-titration study, using a Cd^{2+} selectrode (Radiometer, F3012) in a medium with an ionic strength comparable to that of the assay system (150 mM $NaNO_3$ and 20 mM HEPES/Tris, pH 7.4). The selectrode used is insensitive to Ca^{2+} and Mg^{2+} up to mM concentrations. An association constant $K = pk_d = 5.43 \pm 0.04$ was calculated (Price & Dwek, 1979). Pecoraro, Hermes and Cleland (1984) using ^{32}P NMR (30°C, ionic strength: 0.1 M by addition of KNO_3) found a K value of 4.36 ± 0.28 for Cd-ATP. Although such a difference might be of importance in media with a low buffer capacity for Ca^{2+} and Cd^{2+} it appeared insignificant in our system with 1.5 mM EGTA, HEEDTA plus NTA and 3 mM ATP.

The ^{45}Ca radioactive concentration in the transport medium was 5.6 to 7.4×10^5 Bq · ml^{-1}. The amount of membrane protein per filter was 15 to 20 μg. Filters with retained radioactivity were dissolved in 4 ml Aqualuma® scintillation fluid (15 min at room temperature); the radioactivity was determined in a LKB rack-beta LSC, equipped with a dpm-program.

Vesicle Ca^{2+}-uptake characteristics were similar to those reported by Perry and Flik (1987) for trout gill BLM vesicles. Typically the calcium ionophore A23187 gives release of vesicular $^{45}Ca^{2+}$, indicating that ATP-driven accumulation of Ca^{2+} in the vesicular space occurs. Also, Ca^{2+} uptake proved to be linear for at least 2 min, and this allowed us to perform kinetic analysis on the basis of 1-min incubations. Initial Ca^{2+}-transport velocities were defined as the difference in ^{45}Ca accumulation in the membrane vesicles in the presence and in the absence of ATP.

Table 2. Effect of EGTA treatment and calmodulin repletion on the kinetics of BLM Ca^{2+} transport[a]

	V_{max}[b]	$K_{0.5}$[c]	Corr. coeff.[d]	n
Control	2.26 ± 0.82	0.15 ± 0.06	0.923 ± 0.050	11
EGTA treated	1.58 ± 0.61[e]	0.20 ± 0.07	0.904 ± 0.092	9
EGTA treated *and* CaM repleted	2.14 ± 0.76	0.14 ± 0.07	0.875 ± 0.073	7

[a] EGTA-treated membranes were repleted with 50 mg calmodulin per g protein. The V_{max} and $K_{0.5}$ values were derived from Eadie-Hofstee plots; mean values ± SE are given; n indicates the number of membrane preparations tested.
[b] V_{max} in nmol/min · mg protein.
[c] $K_{0.5}$ in μM.
[d] Correlation coefficients relate to the respective Eadie-Hofstee plots.
[e] $P < 0.03$.

Statistics

Data were statistically analyzed by the Mann-Whitney U-test. Statistical significance was accepted for $P < 0.05$. Linear regression analysis was based on the least-squares method.

Results

Calmodulin Dependency of Ca^{2+} Transport

According to the RIA data the EGTA treatment during membrane isolation resulted in a 34.4% decrease of membrane calmodulin from 3.20 ± 0.10 to 2.10 ± 0.37 mg/g protein ($n = 4$). The effects of this calmodulin depletion on the kinetics of Ca^{2+} transport are shown in Table 2. A significant decrease in the Ca^{2+}-transporting capacity (V_{max}) was observed concomitantly with a decrease in membrane calmodulin content. The affinity of the Ca^{2+} pump for Ca^{2+} decreased (increased $K_{0.5}$), although not significantly. The effects of calmodulin depletion were fully reversed by calmodulin repletion.

Effects of Cd^{2+} on Ca^{2+} Transport

In Fig. 1 the effect of Cd^{2+} on Ca^{2+} transport, at 0.25 μM Ca^{2+}, is shown. Cd^{2+} proved extremely inhibitory towards active Ca^{2+} transport; a 50% inhibition of the Ca^{2+}-transport rate at 0.25 μM was found at 3.0 nM Cd^{2+}.

Kinetic analysis of the Ca^{2+} transport was carried out to establish the nature of inhibition by Cd^{2+}. Figure 2 shows an Eadie-Hofstee plot for Ca^{2+} transport at varying Cd^{2+} concentrations.

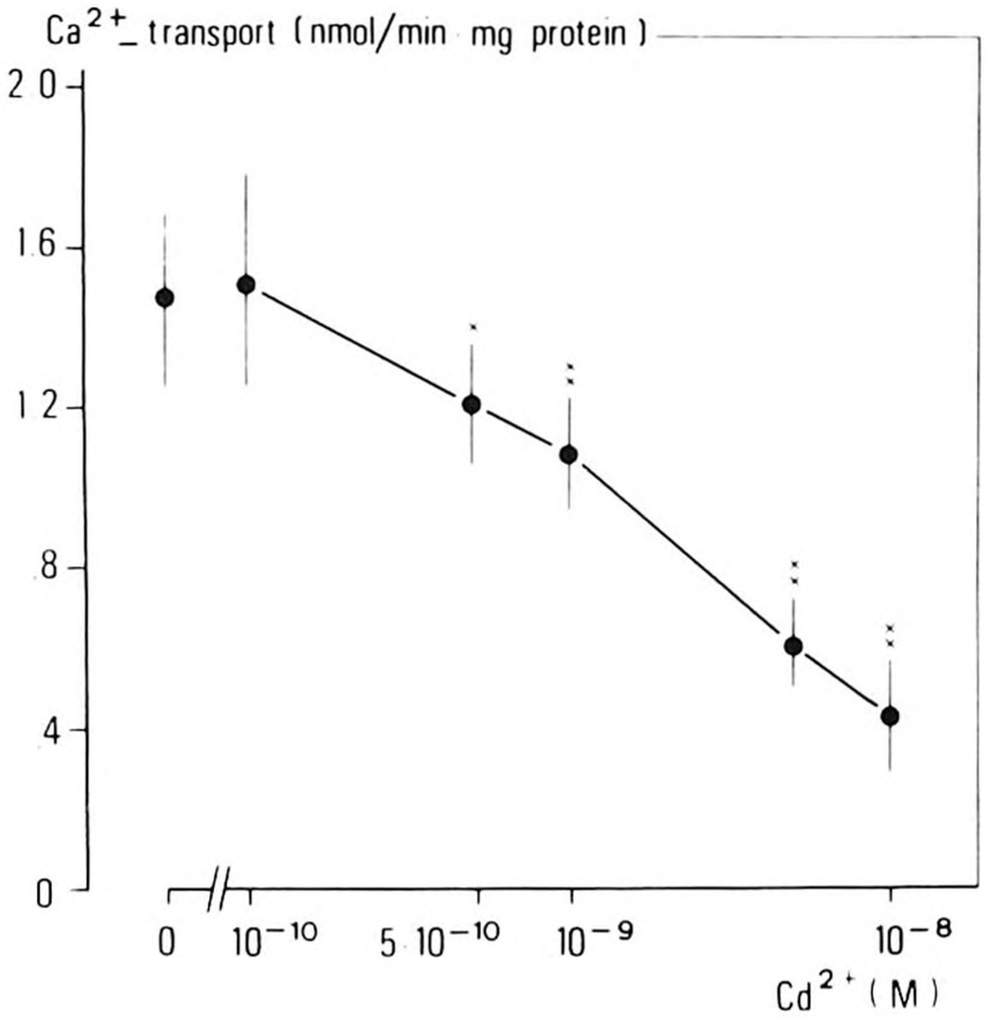

Fig. 1. Inhibition by Cd^{2+} of ATP-dependent Ca^{2+} transport in BLM vesicles of trout gill. The dots represent mean values ± SEM of 1-min uptakes at 0.25 μM free Ca^{2+} from four to eight experiments. x: $P < 0.05$; xx: $P < 0.01$

Fig. 2. Eadie-Hofstee plots of Ca^{2+}-dependency of ATP-dependent Ca^{2+} transport in trout gill BLM vesicles at different free Cd^{2+} concentrations. The dots represent mean values of 1-min uptakes from six experiments

Mean values for the kinetic parameters of the Ca^{2+}-transport process, derived from Eadie-Hofstee transformations of the individual experiments, are given in Table 3. To assure true V_{max} determinations

Table 3. Effect of Cd^{2+} on the kinetics of the BLM Ca^{2+} transport[a]

Cd^{2+} concentrations	V_{max}[b]	$K_{0.5}$[c]	Corr. coeff.[d]
0 (control)	1.99 ± 0.30	0.16 ± 0.05	0.965 ± 0.041
5×10^{-10} M	1.83 ± 0.18	0.17 ± 0.05	0.908 ± 0.074
10^{-9} M	1.92 ± 0.18	0.20 ± 0.06	0.876 ± 0.102
5×10^{-9} M	2.03 ± 0.30	0.93 ± 0.29[e]	0.904 ± 0.074
10^{-8} M	2.23 ± 0.25	1.95 ± 0.25[e]	0.910 ± 0.081

[a] V_{max} and $K_{0.5}$ values were derived from Eadie-Hofstee plots; Ca^{2+} concentrations were varied around the apparent $K_{0.5}$ values. Mean values of six individual observations from different membrane preparations are given.
[b] V_{max} in nmol/min · mg protein.
[c] $K_{0.5}$ in μM.
[d] Correlation coefficients relate to the respective Eadie-Hofstee plots.
[e] $P < 0.005$

at varying Cd^{2+} concentrations, the free Ca^{2+} concentrations were varied around the apparent $K_{0.5}$ values anticipated to occur at the Cd^{2+} concentrations chosen.

From the data in Table 3, a linear increase of $K_{0.5}$ with increasing Cd^{2+} concentration was observed ($r_0 = 0.9995$, $P < 0.001$, $n = 3$). The V_{max} of the Ca^{2+}-transport process was not significantly influenced in the Cd^{2+}-concentration range tested.

Discussion

Four major conclusions are drawn from this study:
1) ATP-driven Ca^{2+} transport in basolateral plasma membranes of gills is extremely sensitive to inhibition by Cd^{2+}.
2) In well-defined assay media that include buffers for Ca^{2+} and Cd^{2+}, only inhibitory effects of Cd^{2+} were observed; stimulatory effects did not occur.
3) The Ca^{2+} pump is calmodulin dependent, but inhibition by Cd^{2+} of the pump is not mediated via this regulatory protein.
4) Kinetic analyses show that Cd^{2+} inhibits the Ca^{2+} pump via its Ca^{2+}-transport site.

Our physiological studies on trout gills have indicated that Cd^{2+} inhibits Ca^{2+} translocation over the basolateral plasma membrane of the chloride cells of the gills (Verbost et al., 1987*a*). In the present study this conclusion is firmly supported by our demonstration that Cd^{2+} inhibits Ca^{2+} transport in basolateral membrane vesicle preparations. The data presented here indicate that Cd^{2+}, due to an extremely high affinity of the Ca^{2+} pump for Cd^{2+}, blocks transepithelial Ca^{2+} passage by inhibition of the BLM Ca^{2+}-ATPase. We speculate that the very

high affinity for Cd^{2+} of the Ca^{2+}-ATPase is indicative of the presence of an SH-(like)group in the Ca^{2+} site of the enzyme.

Transepithelial Ca^{2+} inflow in trout gills depends on the passage of Ca^{2+} from the water to the blood across two membrane barriers, viz. the apical and the basolateral plasma membrane of the chloride cells (Flik et al., 1985*a*). We have shown that the inhibition by Cd^{2+} of the transepithelial Ca^{2+} inflow across the gills does not occur instantaneously, but after an exposure period of several hours. This latency indicates that Cd in the ambient water (in concentrations $<10^{-5}$ M) did not affect the entry of Ca^{2+} in the chloride cells via the apical membrane. In the same study we have shown that Cd^{2+} enters the epithelium, possibly via apical, La^{3+}-inhibitable Ca^{2+} channels. We therefore further concluded that significant buffering of Cd^{2+} occurs in the cytosol of these cells before the Ca^{2+} pump in the BLM becomes inhibited (Verbost et al., 1987*a*).

The present work establishes that trout gill BLM's contain calmodulin. EGTA treatment of the membranes during isolation led only to a 34% calmodulin depletion; apparently this regulatory protein is tightly associated with the membranes. A comparable tight association has been reported for calmodulin in heart sarcolemma (Caroni & Carafoli, 1981). Our approach to calmodulin depletion of BLM resulted in a decrease of the V_{max} of ATP-driven Ca^{2+} transport showing calmodulin dependency of the Ca^{2+} pump. The calmodulin depletion had no significant effect on the enzyme's affinity for Ca^{2+}. Calmodulin antagonists W_7, $C_{48/80}$ and calmidazolium gave only 20% inhibition of Ca^{2+} transport at 100 μM, 100 μg/ml and 1 μM, respectively (after 12-min preincubation on ice or 5-min preincubation at 37°C), indicating poor accessibility of the calmodulin antagonists to the inhibitory sites (*unpublished observations*). Recently Ghijsen et al. (1986) described the same poor accessibility for calmodulin antagonists in plasma membrane vesicles derived from rat duodenum.

A priori at least two possible mechanisms of inhibition of the Ca^{2+} pump should be considered, namely inhibition via its regulator calmodulin and inhibition via the Ca^{2+} site of the enzyme. Cd^{2+} may replace Ca^{2+} on calmodulin (Forsen et al., 1980) and the Cd-calmodulin complex is reported to stimulate calmodulin-dependent enzyme activities (Chao et al., 1984; Flik et al., 1987). On the other hand Akerman et al. (1985) tentatively concluded that the inhibitory effect of Cd^{2+} on calmodulin-activated Ca^{2+},Mg^{2+}-ATPase of human erythrocyte ghosts results from an interaction of the Cd^{2+} ion with calmodulin. We did not find a biphasic effect of Cd^{2+} on ATP-driven Ca^{2+} translocation in trout

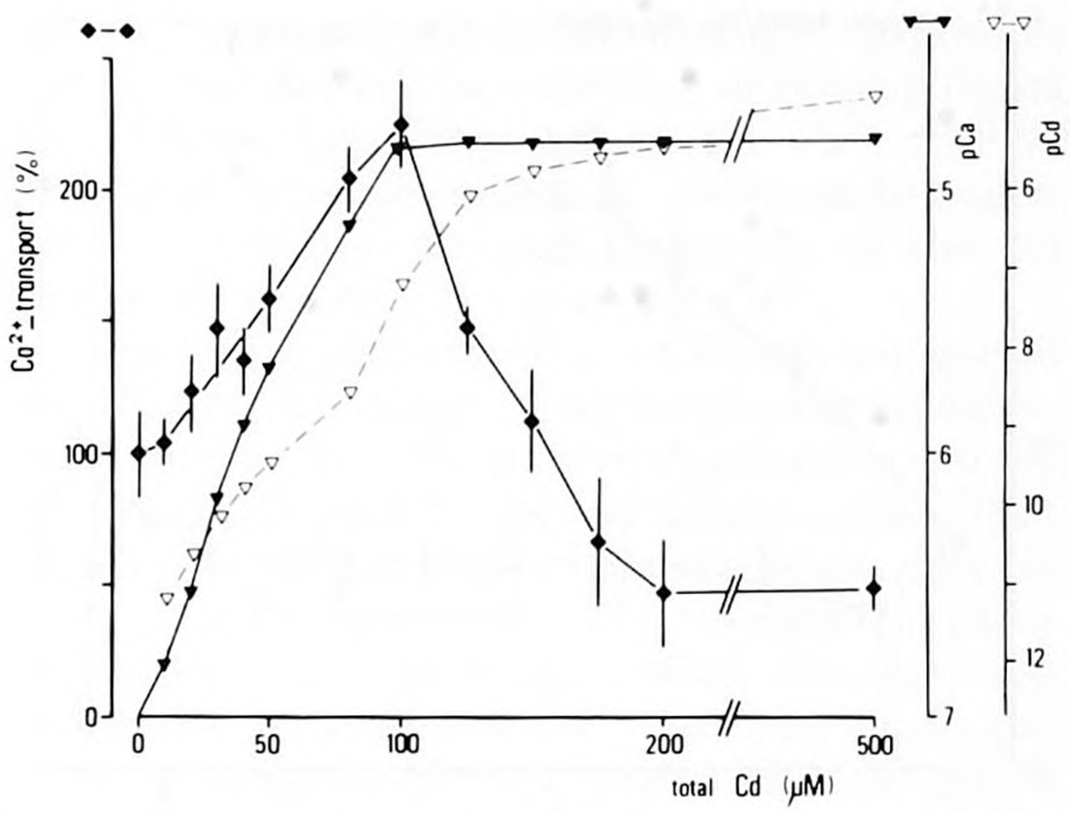

Fig. 3. Effect of addition of graded amounts of Cd to the assay medium on ATP-dependent Ca^{2+} transport in trout gill BLM vesicles. *p*Ca and *p*Cd: the resulting free Ca^{2+} and Cd^{2+} concentrations, calculated to occur at these conditions, are included in the Figure. For this assay a Ca^{2+}/Cd^{2+} buffer of 0.1 mM EGTA plus HEEDTA was used, which is comparable to the buffer conditions chosen in the studies of other Cd-sensitive enzyme systems mentioned in the discussion. Mean values ± SEM for four experiments are given

branchial BLM membrane which seems to distinguish this Ca^{2+}-ATPase from other calmodulin-stimulated enzymes: studies on calmodulin-sensitive Ca^{2+}-dependent myosin light-chain kinase (MLCK; Mazzei et al., 1984) or calmodulin-sensitive phosphodiesterase (Chao et al., 1984; Suzuki et al., 1985; Flik et al., 1987) suggest a stimulatory effect of Cd^{2+} on the enzymes via calmodulin at low Cd concentrations and an inhibitory effect at high Cd concentrations. We feel, however, that an important difference in methodology in our studies and in those of, e.g., Mazzei et al. (1984), Suzuki et al. (1985), and Flik et al. (1987) may underlie this apparent discrepancy. The aforementioned authors reported on the effects of the total Cd concentrations on enzyme activity; in our study we calculated the free Cd^{2+}- and free Ca^{2+}-concentrations in the assay media. The ionic form of Ca^{2+} is generally believed to be the actual physiological effective form; the Cd^{2+} ion is the presumptive toxic form of this heavy metal (Cain & Webb, 1983).

A biphasic effect of Cd on the Ca^{2+} pump is observed when Ca^{2+} transport is determined as a function of total Cd added to the assay system (Fig. 3) using a constant total Ca^{2+} concentration, assuming a constant free Ca concentration of 0.1 μM [the strategy followed by, e.g., Mazzei et al. (1984), Suzuki et al. (1985) and Rauchova, Kaul and Drahota (1985)]. Included in Fig. 3, however, are the actual, calculated free Ca^{2+}- (*p*Ca) and Cd^{2+}-concentrations (*p*Cd). From these calculations we conclude that the Cd-induced enzyme activation at low Cd

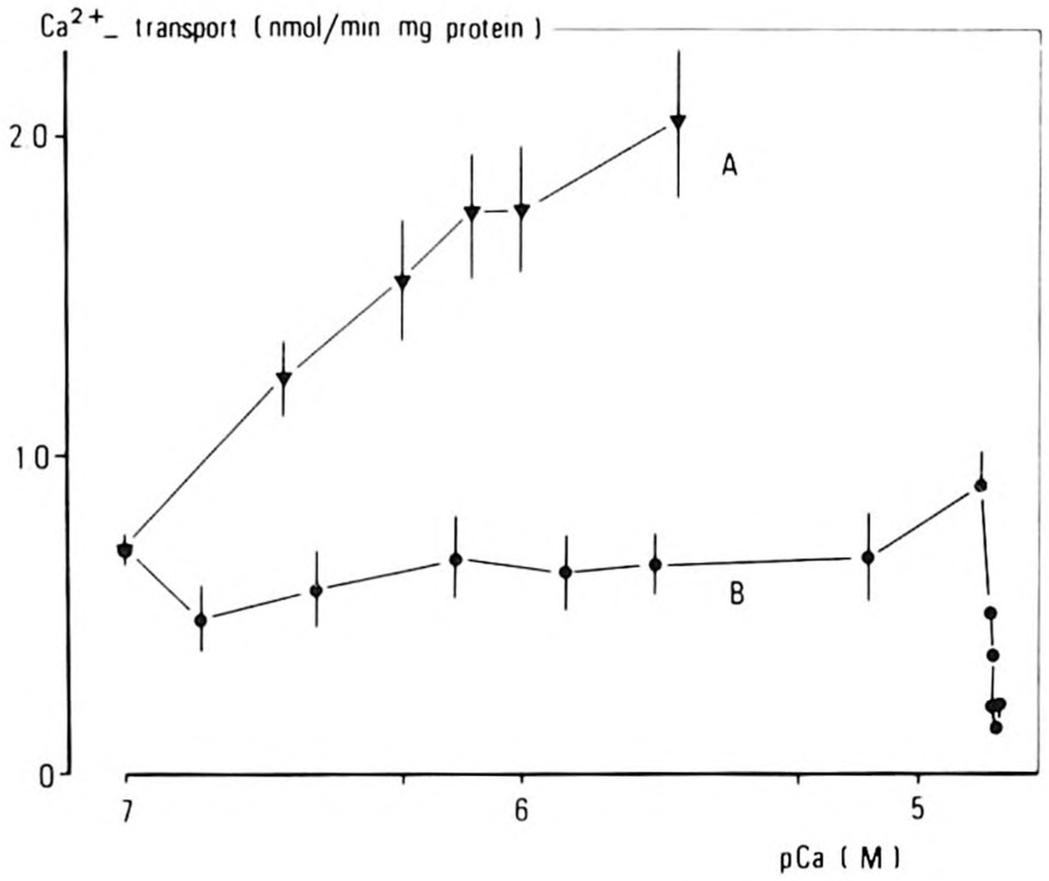

Fig. 4. Line *A* shows the control values for ATP-dependent Ca^{2+} transport in BLM vesicles at different free Ca^{2+} concentrations. Mean values ± SEM for 16 experiments are given. Line *B* represents values derived from Fig. 3 of ATP-dependent Ca^{2+} transport at various total Cd concentrations plotted versus the free Ca^{2+} concentrations calculated to occur at these conditions

concentration is artefactual and is best explained by the increase in the free Ca^{2+} concentration that occurs upon addition of Cd^{2+} to the system (Cd^{2+}, because of its high affinity for the ligands in the media, displaces Ca^{2+} from the ligands). This is illustrated by the fact that at higher Ca^{2+} concentrations with almost no change in free Ca^{2+} upon addition of Cd^{2+}, low Cd^{2+} concentrations only show a progressively inhibitory action. This notion probably explains the results of Mazzei et al. (1984), who reported stimulatory and inhibitory effects of Cd on MLCK and PL-Ca-PK at low and only inhibitory effects at high concentrations of Cd. The fact that Cd stimulates the calmodulin-dependent MLCK as well as the calmodulin-independent PL-Ca-PK invalidates the theory of a stimulation via calmodulin. Another example of "stimulation by Cd^{2+}" of a Ca^{2+}-requiring (and calmodulin-independent) enzyme is that of the mitochondrial glycerol 3-phosphate dehydrogenase: Rauchova et al. (1985) published stimulatory effects at low- and inhibitory effects at high-Cd concentrations. Recalculation of the data given by these authors confirmed our hypothesis that the stimulation of enzyme activity after addition of Cd^{2+} may be the result of an increase in free Ca^{2+}. For example, in the experiments on MLCK by Mazzei et al. (1984) the concentration Ca^{2+} increases 136 times (from 2.2×10^{-7} to 3.0×10^{-5} M), accompanied by an enzyme stimulation, in the concentration range 0 to 50 μM total Cd and with 30 μM total Ca. In the same Cd range with 100 μM total Ca the concentration Ca^{2+} increases only two times (from 5.0×10^{-5} to 9.9×10^{-5} M) and then Cd only caused inhibition of enzyme activity. The concentration Ca^{2+} is 100 to 1000 times higher than the Cd^{2+} concentration in the concentration range 0 to 50 μM total Cd. Moreover, the present results illustrate that the activity of Ca^{2+}-transport ATPase depends on the free Ca^{2+} concentrations in the assay media. We conclude, therefore, that in vitro studies on the actions of Cd^{2+} on Ca^{2+}/calmodulin-dependent enzymes can only be properly carried out in media well defined with respect to metal ion levels.

Besides the fact that the increase in free Ca^{2+} could explain the biphasic character of the effects of Cd on Ca^{2+}-transport activity we wish to emphasize that Cd^{2+} already inhibits Ca^{2+} transport at nanomolar concentrations. In Fig. 4 the Ca^{2+}-transport data from Fig. 3 are plotted versus the Ca^{2+} concentrations that occur upon addition of the various Cd concentrations (line *B*). Line *A* in Fig. 4 represents data on Ca^{2+} transport in the absence of Cd^{2+}. First, we may conclude that even Cd^{2+} concentrations lower than 6×10^{-8} M cause an inhibition of the Ca^{2+} pump. If appropriate corrections for free metal ion levels are omitted, one would erroneously conclude that such concentrations are stimulatory. Secondly, there is a drastically enhanced inhibition of Ca^{2+} transport when the Cd^{2+} concentration exceeds 6×10^{-8} M. We speculate that this inhibition at high Cd^{2+} concentrations results from interactions of Cd^{2+} with other sites of the enzyme than the Ca^{2+}-binding site, for instance the Mg^{2+} site.

Since Suzuki et al. (1985) and Flik et al. (1987) found approximately similar affinities of calmodulin for Ca^{2+} and Cd^{2+}, the calculated molar Cd^{2+}/Ca^{2+} ratio of the media may be used as an indicator of Cd^{2+}-induced calmodulin-mediated effects on the enzyme. The apparently enhanced Ca^{2+}-transport activity resulting from the addition of Cd^{2+} to the Ca^{2+}-transport media (Fig. 3, Ca^{2+} transport > 100%) occurred at Cd^{2+}/Ca^{2+} ratios (Cd^{2+}/Ca^{2+} varied from 4.27×10^{-5} to 11×10^{-2}) at which the formation of significant amounts of Cd-calmodulin complexes seems unlikely. Both the fact that the Ca^{2+} pump is inhibited whenever Cd^{2+} is present and the fact that no significant amounts of Cd-calmodulin will occur in the media make a stimulatory action of Cd^{2+} via calmodulin unlikely.

Our results contradict the results of Akerman et al. (1985) who concluded that Cd^{2+} indirectly (via calmodulin) inhibits the Ca^{2+},Mg^{2+}-ATPase from erythrocyte ghosts. The calculated free Cd^{2+} concentration in their media was 1.2 to 1.4×10^{-11} M (and the Cd^{2+}/Ca^{2+} concentration ratio calculated on the basis of their data varied from 5.5×10^{-3} to

5.5×10^{-6}) which makes the formation of significant amounts of Cd-calmodulin complexes unlikely and the interpretation of their data very difficult.

Kinetic analysis showed that inhibition of the plasma membrane Ca^{2+}-transport process by Cd^{2+} is competitive, as shown by a linear increase of $K_{0.5}$ with increasing Cd^{2+} concentration and no significant effect on V_{max}. The competitive nature of inhibition of the Ca^{2+}-transport process by Cd^{2+} together with the unlikely occurrence of Cd-calmodulin complex formation (*see above*) provides evidence against an inhibition of the Ca^{2+} pump by Cd^{2+} via calmodulin (at least at Cd^{2+} concentrations $<10^{-8}$ M).

We conclude that Cd^{2+} inhibits Ca^{2+} transport in BLM by occupying the Ca^{2+}-transport site of the Ca^{2+}-ATPase. At the moment we can only speculate about intracellular free Cd^{2+} concentrations that may occur in the gill epithelium in vivo. We calculated that the gill tissue Cd content is 13.2 μmol/kg wet weight tissue after a 45-min exposure to 1 μM Cd in the water. The free Cd^{2+} concentration may be expected to be around 1.5×10^{-7} M if one takes into account a 0.15 mM Ca-binding protein buffer in the cytosol (Van Os, 1987). In view of the extremely high affinity for Cd^{2+} of Ca^{2+}-pumping ATPases in vitro it seems reasonable to consider that Cd^{2+}, even though it may be bound to intracellular Ca^{2+} receptors for a greater part, still may have an inhibitory effect on the Ca^{2+} transport and by so doing eventually raise cytosolic Ca^{2+} levels.

An important question is whether the active Ca^{2+} transport is the most sensitive target for a cell being intoxicated by Cd^{2+}. It may be that inhibition of Ca^{2+} transport leads to other disturbances or that it is only one aspect of Cd^{2+} toxicity for the cell. Nechay and Saunders (1977) working with rat kidney cortex microsomes showed an inhibitory effect of Cd on Na^+,K^+-ATPase activity. However, the free Cd^{2+} concentration causing 50% inhibition of enzyme activity was very high (3 μM free Cd^{2+}) as we calculated on the basis of their data (0.26 mM total Cd, 3 mM ATP, 3 mM Mg). Sugawara and Sugawara (1975) reported that Cd^{2+} inhibited rat intestinal brush-border alkaline phosphatase activity in vitro; even in the presence of 100 μM Cd^{2+} this activity was still as high as about 50% of the control value. Compared to these results Ca^{2+} extrusion over the plasma membrane seems to be the most Cd^{2+}-sensitive process reported so far. This supports the idea that Cd^{2+} interferes specifically with Ca^{2+}-ATPases that extrude Ca^{2+} from the cytosol and, as a secondary effect, upsets other cellular events. For renal cells it was recently demonstrated that cytosolic Ca^{2+} increased after Cd^{2+} administration to rats (Maitani, Watahiki & Suzuki, 1986). Also the hypocalcemia in fish after Cd^{2+} exposure, that is accompanied by inhibition of transepithelial Ca^{2+} inflow (which depends on the Ca^{2+}-ATPase activity in the tissue; Flik et al., 1984) can be understood in view of the high sensitivity of the gill plasma membrane Ca^{2+} pump for Cd^{2+}.

Our conclusion that Ca^{2+}-ATPases are one of the primary Cd^{2+} targets contributes to the interpretation of Cd^{2+} toxicology. Investigations on rat enterocyte BLM vesicles and rat kidney cortex BLM vesicles revealed a very similar inhibitory effect of Cd^{2+} on ATP-dependent Ca^{2+} transport in these membranes (Verbost et al., 1987*b*). We have suggested that all membrane Ca^{2+}-ATPases which also occur in Golgi membranes and RER, and play a dominant role in cellular Ca^{2+} homeostasis, may be inhibited at nanomolar Cd^{2+} concentrations.

This study was supported by a grant from the Foundation for Fundamental Biological Research (BION), which is subsidized by the Dutch Organization for the Advancement of Pure Research (ZWO). The authors thank Mr. D. Mulder for determining the affinity constant for Cd-ATP.

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Received 29 June 1987; revised 7 January 1988